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PRINCIPAL INVESTIGATOR: Kermit L. Carraway, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Inc.
Boston, MA 02215

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Ascites 13762 cells, a highly metastatic and malignant rat mammary adenocarcinoma cell line, express a cell surface glycoprotein called ASGP2 that possesses two domains believed to interact with and stimulate growth factor receptors of the ErbB family. These cells also express p185$^{erbB2/neu}$, a member of the ErbB family whose aberrant activation probably plays a role in tumorigenesis and tumor progression in a significant subset of breast cancer patients. ASGP2 and p185$^{erbB2/neu}$ exist in a complex at the surface of the ascites 13762 cells, raising the possibility that these cells stimulate their own growth or malignancy through an autocrine mechanism involving the ASGP2/p185$^{erbB2/neu}$ interaction. The overall goal of the studies outlined in this proposal is to test this hypothesis. Studies are aimed at examining the effect of ASGP2/p185$^{erbB2/neu}$ complex expression on the growth and transformation of cultured mammalian cells, including human breast cancer cells. We have attempted to stably express ASGP2 in a variety of cell lines and have found only one, the human mammary tumor cell line MCF7, that is capable of supporting the stable expression of this protein. Studies are currently underway to characterize the growth properties of transfectants.

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Introduction

Epidermal growth factor (EGF) is the prototype for a family of ligands that influence not only the proliferation and differentiation of cells in normal tissues but also the growth and possibly the malignancy of cancer cells (1,2). Stimulation of normal cells and tissues by the EGF-like growth factors generally proceeds through endocrine or paracrine mechanisms, where the target cell is in a location distal to the cells secreting the diffusible growth factor. However, accumulating evidence indicates that autocrine, juxtacrine and intracrine mechanisms may also play roles in cellular growth regulation, particularly for tumor cells.

The EGF-like ligands exert their effects through the ErbB family of growth factor receptor/tyrosine kinases, which includes the EGF receptor, $p185^{erbB2/neu}$, $p180^{erbB3}$ and $p180^{erbB4}$ (3). Overexpression of each of the members of the ErbB receptor family has been observed in human tumors, and it is suspected that the activated receptors participate in tumor formation and progression. Particular attention has focused on $p185^{erbB2/neu}$ because the aberrant activation of this protein is known to be tumorigenic to transgenic animals. More importantly, the amplification of the *erbB2/neu* gene and the overexpression of the $p185^{erbB2/neu}$ product has been observed in a significant proportion of breast cancer patients. In fact, several studies suggest that the overexpression of $p185^{erbB2/neu}$ correlates with a poor patient prognosis for some subsets of patients (4-6). Since overexpression of the $p185^{erbB2/neu}$ protein leads to its increased kinase activity, these observations suggest that the aberrant activation of $p185^{erbB2/neu}$ kinase activity directly contributes to breast cancer tumorigenesis and progression. Thus far, most studies concerning $p185^{erbB2/neu}$ and breast cancer have been directed toward characterizing the amplification and overexpression of the *erbB2/neu* gene in tumors. The goal of such studies was to develop reagents that might prove useful in the diagnosis of malignant forms of breast cancer, based on the overexpression of the $p185^{erbB2/neu}$ protein (4-6). It is clear, however, that overexpression of $p185^{erbB2/neu}$ is only one of possibly many mechanism for its activation. Indirect activation of $p185^{erbB2/neu}$ by EGF and HRG through heterodimerization with and cross-phosphorylation by other ErbB family members represents another mechanism for activation. These observations raise the question of whether or not a direct ligand activator for $p185^{erbB2/neu}$ exists. Several activities have been reported but none have been cloned or characterized in detail. Most screens for activating ligands use either serum or (7-9) conditioned media from cultured cells as starting material. However, given the diversity in mechanisms by which growth factors can act (juxtacrine, intracrine, etc.) it is possible that some activating ligands do not act as secreted factors, but are only active within the membrane environment. Because such proteins would possess hydrophobic transmembrane sequences, they may be difficult to identify, purify and characterize. However, such ligands could be potent activators of $p185^{erbB2/neu}$ activity, and may be intimately involved in the contribution of $p185^{erbB2/neu}$ to breast cancer.

Ascites 13762 cells are an autonomously proliferating, highly metastatic and malignant rat mammary adenocarcinoma cell line propagated in the rat peritoneal cavity. These cells express in abundance at their surfaces a complex of two glycoproteins, the ascites sialoglycoproteins ASGP1 and ASGP2 (10). ASGP1 is a sialomucin of molecular weight >600 kDa, over 400 kDa of which is O-linked oligosaccharide (11). Its abundance, large size and heavy glycosylation allows it to serve as an inhibitor of protein-protein and cell-cell interactions, as well as a screen from natural killer cells of the immune system (12-14). Although synthesized from the same message, ASGP2 has a very different character. It is a ~120 kDa glycoprotein consisting of predominantly N-linked glycosyl moieties, and is the membrane-bound component of the complex (15). Upon cloning and sequencing of the complex (16), it was found that ASGP2 possesses at least five identifiable subdomains: a very small intracellular domain of 20-25 amino acids, a single hydrophobic transmembrane domain, a cysteine-rich domain of unknown function, and two EGF-like domains that contain all the consensus amino acid residues characteristic of the active growth factors. Moreover, the spacing of cysteine residues in the EGF-like domain closer to the amino terminus of the protein (N-EGF) is very similar to those of the active growth factors.

These observations raised the possibility that the ASGP2 protein is capable of binding to and activating receptors of the ErbB family. Using insect cell technology, we have shown that ASGP2 selectively binds to $p185^{erbB2/neu}$, that the N-EGF domain mediates this interaction, that the co-expression of the two proteins within the same cell significantly facilitates their stable interaction, and that purified ASGP2 stimulates the tyrosine kinase activity of $p185^{erbB2/neu}$ (see Appendix). Our observations indicate that the ASGP2 and $p185^{erbB2/neu}$ proteins are capable of forming a complex in cells that co-express the two proteins, and that this association can result in the stimulation of $p185^{erbB2/neu}$ autophosphorylation and tyrosine kinase activities. Considering that the aberrant activation of $p185^{erbB2/neu}$ is suspected to be involved in breast cancer progression, our observations point to the possibility that the expression of ASGP2 by breast cancer cells might contribute to their autonomous growth or malignancy by stimulating $p185^{erbB2/neu}$ activity. We propose that ASGP2 can act as an autocrine or intracrine activating ligand for the $p185^{erbB2/neu}$ receptor in cells that co-express the two proteins, and that the expression of the ASGP2/$p185^{erbB2/neu}$ complex stimulates cellular growth properties. The overall goal of the studies discussed below is to rigorously test this hypothesis by introducing the complex into cultured cells, and determining whether the cellular growth state is significantly influenced.

Results and Discussion

Specific Aim I: Analyze the capacity of ASGP2 to act as an activating ligand for the $p185^{erbB2/neu}$ receptor when the two proteins are co-expressed in cultured mammalian cells.

On the basis of our previous observations, we propose that the co-expression of ASGP2 with $p185^{erbB2/neu}$ enhances the tyrosine kinase activity and autophosphorylation of the receptor. Our previous attempts to test this hypothesis in insect cells were unsuccessful because insect cell-expressed $p185^{erbB2/neu}$ is constitutively active and fully autophosphorylated. To try to circumvent this problem, we have employed a mammalian expression system to assess the extent to which ASGP2 stimulates $p185^{erbB2/neu}$ tyrosine phosphorylation. COS7 monkey cells were transiently transfected with plasmids directing the constitutive expression of ASGP2, $p185^{erbB2/neu}$, or the two proteins together. The association of the two proteins was assessed by co-immunoprecipitation experiments, and the extent of $p185^{erbB2/neu}$ tyrtosine phosphorylation was assessed by anti-phosphotyrosine immunoblotting.

For these experiments, the cDNA encoding rat $p185^{erbB2/neu}$ was introduced using the pSV2 vector, which allows expression under the constitutive RSV promoter. The cDNA encoding rat ASGP2 was introduced using the pcDNA3 vector, which allows expression under the constitutive CMV promoter. The cDNA encompassing the coding region of ASGP2 was fused in-frame with the leader sequence for cathepsin D to facilitate the membrane localization of the ASGP2 protein. Cells were transfected using the Lipofectamine reagent (Gibco) according to the directions of the manufacturer. 48 and 72 hours following transfection, cells were lysed according to our previous protocols, and lysates were immunoprecipitated with anti-ASGP2 or anti-$p185^{erbB2/neu}$ antibodies. Precipitates were analyzed by SDS-PAGE followed by Western blotting for co-precipitating proteins. In some experiments, lysates were immunoblotted with anti-phosphotyrosine antibodies to determine whether the presence of ASGP2 impacted the extent of $p185^{erbB2/neu}$ autophosphorylation.

The results obtained using COS cells were essentially identical to those that we obtained previously using the insect cell expression system. We observed that ASGP2 could be co-precipitated with anti-$p185^{erbB2/neu}$ in co-transfected COS cells. Likewise, $p185^{erbB2/neu}$ could be co-precipitated with anti-ASGP2 in co-transfecteants. Also like with the insect cells, we observed a very high basal level of $p185^{erbB2/neu}$ autophosphorylation, even in the absence of the co-expressed ASGP2. Co-expression of the putative ligand did not increase the level of tyrosine phosphorylation of $p185^{erbB2/neu}$. In fact, cells that had been co-

transfected displayed a lower level of $p185^{erbB2/neu}$ tyrosine phosphorylation than did cells expressing $p185^{erbB2/neu}$ alone. Upon re-probing of the blots with anti-$p185^{erbB2/neu}$ we determined that this was due to a reduced level of $p185^{erbB2/neu}$ protein in co-transfectants. Although we did not observe an effect of ASGP2 on $p185^{erbB2/neu}$ tyrosine phosphorylation, these observations are consistent with a ligand-like behavior of ASGP2 toward $p185^{erbB2/neu}$ in that it appears to down-regulate the expression of the receptor.

The problem with these studies appears to be that the levels of $p185^{erbB2/neu}$ expression in transfected cells is too high, allowing its constitutive activation. We have attempted to vary the parameters of transfection to vary the transfection efficiency, and have reduced the times of transfection to suppress the level of $p185^{erbB2/neu}$ expression within a transfected cell. However, when either of these parameters are varied the level of $p185^{erbB2/neu}$ expression decreases dramatically such that in most instances it cannot be detected at all. For these reasons we have diverted our attentions to mammalian cells that constutively express modest levels of $p185^{erbB2/neu}$. Receptor autophosphorylation in such cells is very low in the absence of ligand, and offers the highest chance of observing a stimulation by stably co-expressed ASGP2. Also, the co-localization studies proposed for the upcoming year may be carried out with such a system as easily as the with the COS expression system.

Specific Aim II: Determine whether the expression of the ASGP2/$p185^{erbB2/neu}$ complex influences cellular growth or transformation.

We propose that the expression of the ASGP2/$p185^{erbB2/neu}$ complex by ascites 13762 cells stimulates their autonomous growth. The ultimate goal of this proposal is to determine whether or not this is plausible by expressing the complex in model cells and determining its effect on various cellular growth properties. Previous studies have demonstrated that activation of the kinase activity of $p185^{erbB2/neu}$ can lead to mitogenesis in or transformation of rodent fibroblasts (17,18-22). Likewise, some human mammary carcinoma cell lines proliferate in response to HRG, an indirect activator of $p185^{erbB2/neu}$ (23). On the other hand, other human mammary tumor cell lines appear to differentiate in response to HRG treatment (24), and when introduced into PC12 cells, activated $p185^{erbB2/neu}$ induces cellular differentiation (25,26). Hence, activation of the kinase activity of $p185^{erbB2/neu}$ can lead to a variety of responses depending on the cellular context. The goal of the studies described for this Aim is to examine the effect of the ASGP2/$p185^{erbB2/neu}$ complex on cellular growth properties by introducing the complex into different cell types and measuring properties associated with growth state.

To address these issues, cell lines had to be established that express the ASGP2 protein. For the reasons mentioned above, of particular interest were cells that express modest levels of the $p185^{erbB2/neu}$ protein. Also of interest were fibroblast cell lines that overexpress $p185^{erbB2/neu}$. The $p185^{erbB2/neu}$ in such cells have a high constitutive kinase activity, but the cells are not phenotypically transformed. Hence, a stimulation of activity should induce their transformation.

We began our studies by using the transient transfection protocols described above on DHFR/G8 cells. These are mouse fibroblasts that stably express very high levels of $p185^{erbB2/neu}$, but are not transformed. We transfected these cells with a plasmid expressing ASGP2 under the constitutive CMV promoter. After 14 days we examined dishes of cells for the presence of foci, indicative of cellulr transformation. We observed no focus formation in cells transfected with ASGP2 or control plasmid, but did observe focus formation when cells were transfected with a plasmid encoding the activated mutant form of rat $p185^{erbB2/neu}$. Hence, either ASGP2 was not expressed at appropriate levels to stimulate cellular transformation in our experiments, or ASGP2 is incapable of synergizing with $p185^{erbB2/neu}$ to mediate cellular transformation.

At the same time we were stably transfecting a variety of cell lines with ASGP2. We used our standard transfection protocol to introduce ASGP2 under the CMV promoter into NIH3T3 and DHFR/G8 rodent

fibroblasts, and into the human mammary tumor cells MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, MCF7 and SKBR3. A431 human cervical carcinoma cells and A549 human lung carcinoma cells were also transfected. For each transfection, drug-resistant colonies were selected (our plasmid confers resistance to the G418 antibiotic), expanded and screened for ASGP2 expression by immunoblotting whole cell lysates. We observed that only one cell line, the MCF7 human mammary carcinoma cell line, was capable of supporting the stable expression of ASGP2. Colonies derived from the other cell lines exhibited no detectable expression, perhaps because they have a propensity to degrade the form of the protein that we have expressed.

We are currently examining the growth properties of the MCF7 transfectants using [3H]thymidine uptake experiments to measure cellular growth rates. While these studies are still in progress, qualitatively we have observed no significant difference in the overall growth rate in 10% serum or the morphology of transfectants relative to the parental cells. Question under investigation now are whether ASGP2 influences the growth rate of serum-starved cells, or influences their growth response to the heregulin indirect $p185^{erbB2/neu}$ ligand. We are also examining the biochemical properties of these cells. We have observed that as in the insect cells and the co-transfected COS cells, ASGP2 and $p185^{erbB2/neu}$ may be co-immunoprecipitated, indicating that they are forming a complex. We have also observed that in the ASGP2-expressing cells, the basal level of tyrosine phosphorylation of a protein in the size range of the ErbB receptors is increased. Whether this protein is $p185^{erbB2/neu}$ remains to be determined. We have also noted that the mobility on SDS gels of $p185^{erbB2/neu}$ from ASGP2-transfected cells is different from that of the parental cell line, suggesting that ASGP2 is altering the total phosphorylation of the $p185^{erbB2/neu}$ protein. Tryptic peptide mapping experiments are in progress to determine whether there are differences in the sites of $p185^{erbB2/neu}$ phosphorylation when cells do and do not co-express ASGP2. Studies are just now being initiated to develop methods to examine the localization of $p185^{erbB2/neu}$ and ASGP2 in the parental and ASGP2-transfected MCF7 cells. These experiments should reveal whether or not ASGP2 influences the cellular localization of $p185^{erbB2/neu}$.

Conclusions

The goal of these studies is to determine whether ASGP2 interaction with $p185^{erbB2/neu}$ influences the phosphorylation state of the receptor, and influences the growth state of $p185^{erbB2/neu}$-expressing cells when ASGP2 is co-expressed. Initially, these studies were slowed because of the very high level of basal $p185^{erbB2/neu}$ phosphorylation when ectopically introduced into cells. However, this problem could be solved by introducing ASGP2 into a cell line that constitutively expresses very modest levels of the $p185^{erbB2/neu}$ protein. While considerable time and effort was put into transfecting a number of such cell lines, we finally found stable ASGP2 transfectants of a human mammary tumor cell line, MCF7. These cells express very modest levels of $p185^{erbB2/neu}$, whose tyrosine phosphorylation may be stimulated in an essentially all-or-none manner by the indirect ligand heregulin. We feel that we are now in a position to address all of the proposed questions concerning ASGP2 effects on $p185^{erbB2/neu}$ kinase activity and localization, as well the influence of ther ASGP2/$p185^{erbB2/neu}$ complex on cellular growth properties.

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APPENDIX

An Intramembrane ErbB2/Neu Ligand Expressed in a Metastatic Mammary Tumor

Kermit L. Carraway III*, Edmund A. Rossi, Maria E. Carvajal, Pamela M. Guy, Lewis C. Cantley, Nevis Fregien, Coralie A. Carothers Carraway and Kermit L. Carraway

Kermit L. Carraway III and Lewis C. Cantley, Department of Cell Biology, Harvard Medical School, and Division of Signal Transduction, Beth Israel Hospital, Boston, MA 02215

Edmund A. Rossi, Maria E. Carvajal, Nevis Fregien and Kermit L. Carraway, Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33101

Pamela M. Guy, Department of Medicine, University of California, San Diego, La Jolla, CA 92093

Coralie A. Carothers Carraway, Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL 33101

*Address correspondence to:
Kermit L. Carraway III
Beth Israel Hospital
Harvard Institutes of Medicine, Room 1018
330 Brookline Avenue
Boston, MA 02215
Phone (617) 667-0934
FAX (617) 667-0957
E-mail kcarrawa@mercury.bih.harvard.edu

Constitutive activation of the ErbB2/Neu receptor tyrosine kinase may contribute to the progression of breast and ovarian tumors. ErbB2/Neu may be activated indirectly by ligands for other members of the ErbB family of receptors through receptor heterodimerization mechanisms, but no direct ligand for ErbB2 has been molecularly characterized. ASGP2, a transmembrane glycoprotein found on the surface of the highly metastatic ascites 13762 rat mammary adenocarcinoma cell line, possesses an EGF-like domain that shares the consensus residues for activating ligands of the ErbB receptor family. ASGP2 is constitutively associated with ErbB2/Neu at the surface of 13762 cells, and purified ASGP2 stimulates ErbB2/Neu tyrosine kinase activity *in vitro*. Expression studies indicate that the ligand and receptor must be expressed in the same cell for their direct stable interaction to occur. These observations implicate a novel intramembrane mechanism for tumor cell growth regulation through ErbB2/Neu.

ErbB2/Neu is a 185 kD cell surface transmembrane receptor tyrosine kinase which mediates the growth or differentiation of a variety of cultured cells and contributes to the proper development of cardiac and neural tissues during gestation (1). Its overexpression in numerous human tumors, including breast and ovarian tumors, correlates with earlier patient relapse and poor prognosis (2). The observation that ErbB2/Neu overexpression stimulates its protein tyrosine kinase activity, together with the observation that activated alleles of the *erbB2/neu* gene induce metastatic tumors when expressed in murine mammary epithelium (3), suggest that stimulation of ErbB2/Neu kinase activity may play an important role in tumorigenesis or tumor progression.

The protein tyrosine kinase activity of ErbB2/Neu may be activated by several soluble, diffusible ligands which possess epidermal growth factor (EGF)-like domains. For example, EGF, transforming growth factor-α (TGF-α) and amphiregulin (AR) are all capable of stimulating ErbB2/Neu activity by binding to the related EGF receptor and promoting its heterodimerization with ErbB2/Neu (4). Likewise, the neuregulins (NRGs) bind to the ErbB3 and ErbB4 receptors and stimulate ErbB2/Neu activity through receptor heterodimerization mechanisms (5). Since no characterized diffusible ligand has been reported to act on ErbB2/Neu directly, it has been suggested recently that this receptor may not have an intrinsic activating ligand and functions as a heterodimerizing co-receptor for other members of the ErbB family (6). However, this suggestion does not take into consideration the possibility that intracrine or membrane-bound ligands for this receptor have gone undetected because previously employed methodologies would have identified only diffusible factors.

The autonomously proliferating and highly metastatic rat ascites 13762 mammary adenocarcinoma cell line expresses a large sialomucin complex in abundance at its cell surface. This complex consists of two non-covalently associated proteins, ASGP1 and ASGP2, which arise from the proteolytic processing (7) of the product of a single gene (8). ASGP1, a ~600 kD heavily O-glycosylated sialomucin, is an anti-adhesive factor as well as a contributor to the ability of these

cells to evade immune recognition (9). The 120 kD transmembrane subunit ASGP2 tethers the complex to the cell surface. The sequence of ASGP2 includes two EGF-like domains, one of which conserves all of the consensus residues of the active EGF-like growth factors (8). 13762 ascites cells also express at their surfaces modest levels of ErbB2/Neu. In contrast to cell lines that express similar receptor levels, such as rodent fibroblasts or MCF7 human mammary carcinoma cells, ErbB2/Neu in 13762 cells appears to be constitutively tyrosine phosphorylated (10).

To test the hypothesis that ASGP2 might act as a binding ligand for ErbB2/Neu in 13762 cells, the association of these two proteins in membranes was first examined by sedimentation (11). Sucrose density fractionation of solubilized microfilament-depleted 13762 cell microvillar membranes yielded two peaks of co-migrating ASGP2 and ErbB2/Neu (Fig. 1A). The faster-sedimenting peak near the bottom of the gradient (fractions 1-2) contained the actin-associated ErbB2/Neu-containing signal transduction particle described previously (12); the slower-sedimenting peak (fractions 8-11) contained solubilized monomeric membrane proteins and complexes not associated with the signaling particle. ErbB2/Neu migrated at the leading edge of this peak, indicating that it exists in a complex larger than the ~700 kD sialomucin complex. Since no ErbB2/Neu was detected at the very top of the gradient (fraction 11), these observations suggest that all of the ErbB2/Neu in these membranes exist in large multimeric complexes that co-fractionate with the ASGP2-containing sialomucin complex. ASGP2 in fraction 11 represents free sialomucin complex with no ErbB2/Neu bound, consistent with its greater abundance in 13762 cells (7).

To determine whether ASGP2 and ErbB2/Neu are physically associated in 13762 membranes, fractions 8-10 from the gradient were pooled and immunoprecipitated with either anti-ASGP2 or anti-ErbB2/Neu (13), and precipitates were immunoblotted with antibodies to the other. ASGP2 was observed in anti-ErbB2/Neu immunoprecipitates, and ErbB2/Neu was observed in anti-ASGP2 precipitates (Fig. 1B). Since the immunoprecipitations were performed using fractions

isolated from microvillar membranes, these findings indicate that ASGP2 and ErbB2/Neu are present in a complex on the surface of 13762 cells. When taken with the sedimentation data, the co-immunoprecipitation results imply that all of the cell surface ErbB2/Neu in 13762 cells is bound to ASGP2. Interestingly, during pregnancy the expression of ASGP2 in the mammary epithelium of rats increases dramatically, and a fraction of the expressed ASGP1/ASGP2 sialomucin complex is secreted into milk. As with the 13762 cells, ASGP2 and ErbB2/Neu can also be co-immunoprecipitated from lysates of homogenized mammary tissue from pregnant animals (14), suggesting that their association is a normal physiological event and not a result of the aberrant expression of ASGP2 in the tumor cells.

The observations above indicate that ASGP2 is constitutively associated with ErbB2/Neu at the surface of 13762 cells, consistent with an intramembrane mechanism for ligand/receptor interaction. However, the participation of another receptor or other proteins in the binding process could not be ruled out. To address these questions, a baculovirus/insect cell expression system was developed to test key aspects of the intramembrane hypothesis. Insect cells were employed because they do not express endogenous ErbB receptors, eliminating confusion arising from potential receptor heterodimerization events. In the first series of experiments, Sf9 insect cells were infected with baculovirus encoding a transmembrane form of ASGP2 ($ASGP2_{tm}$) alone, EGF receptor alone or ErbB2/Neu alone, or co-infected with $ASGP2_{tm}$ and each of the receptors (15). The co-imunoprecipitation assay was used to assess association. It was observed that $ASGP2_{tm}$ could be co-immunoprecipitated with ErbB2/Neu from cells expressing both proteins, but could not be co-precipitated with the EGF receptor (Fig. 2A). Likewise, ASGP2 could be co-immunoprecipitated with ErbB2/Neu when the two proteins were co-expressed in COS monkey cells, but could not be co-precipitated with the endogenous COS cell EGF receptor (16). These observations indicate that the stable association of ASGP2 with ErbB receptors tends to be selective for ErbB2/Neu, and does not require another ErbB receptor. Deletion analysis indicated that, as expected, the extracellular domain of ErbB2/Neu is necessary for its interaction with

membrane-bound ASGP2. When co-expressed in SF9 cells, ASGP2 could be co-immunoprecipitated with either full-length ErbB2/Neu or the extracellular domain of the receptor, but could not be co-precipitated with the intracellular domain or a transmembrane form lacking the bulk of the extracellular domain (Fig. 2B).

The extracellular domains of the ErbB2/Neu and ASGP2 proteins could also be secreted as a complex when co-expressed in the same cell. In the experiment shown in Fig. 3A, High Five insect cells (cells specifically adapted for the expression of secreted proteins) were infected with baculovirus encoding the extracellular domain of ASGP2 (ASGP2 ECD) or the extracellular domain of ErbB2/Neu (Neu ECD), or were co-infected with both viruses (17). The co-immunoprecipitation assay was then carried out with the cleared conditioned media from infected cells using anti-ErbB2/Neu antibodies. ASGP2 ECD was detected in immunoprecipitates from cells expressing both proteins, indicating that the cells secrete ASGP2 ECD and Neu ECD as a complex. Similar immunoprecipitates from metabolically labeled cells showed no other detectable radiolabeled bands (18), suggesting that the ASGP2-ErbB2/Neu association occurs through a direct protein-protein interaction. Moreover, resolution of the radiolabeled immunoprecipitated proteins by non-reducing SDS-PAGE demonstrated that the association of ASGP2 and ErbB2/Neu is non-covalent in the secreted complex from the High Five cells. Finally, sedimentation analysis of the secreted complex suggests that ASGP2 is capable of associating in a 1:1 complex with monomeric Neu ECD (18).

To determine the domain within ASGP2 which mediates its interaction with ErbB2/Neu, deletion mutagenesis from the carboxy terminus of the ligand was performed. ASGP2 deletion mutants were co-expressed with Neu ECD in High Five cells, and the co-immunoprecipitation assay was employed to determine the extent of interaction between the expressed secreted proteins (Fig. 3B). ASGP2 forms containing EGF1, the EGF-like domain that possesses the consensus residues found in active growth factors (8), could be co-immunoprecipitated with ErbB2/Neu ECD.

However, when the EGF1 domain was deleted, the ability of ASGP2 to associate with ErbB2/Neu was almost completely abolished. These results indicate that, as predicted, the EGF1 domain of ASGP2 is required for its stable interaction with the ErbB2/Neu receptor.

The results above indicate that ASGP2 acts as a direct binding ligand for the ErbB2/neu receptor when the two proteins are co-expressed in the same cell. However, receptor activation experiments could not be carried out in insect or COS cells because the high basal autophosphorylation of ErbB2/neu masked ligand activation. To examine the functional outcome of the interaction, purified ASGP2 (19) was used to stimulate the tyrosine kinase activity of ErbB2/Neu in membranes. It was observed that in partially solubilized purified plasma membranes from DHFR/G8 cells (20), mouse fibroblasts transfected with the rat *erbB2/neu* cDNA, ASGP2 but not EGF promoted the phosphorylation of the ErbB2/Neu protein (Fig. 4A). ASGP2 also stimulated the tyrosine kinase activity of the membranes toward the exogenous synthetic substrate poly(Glu_4Tyr). These results suggest that ASGP2 acts as an activating ligand for ErbB2/Neu.

Fig. 3A demonstrates that the co-expression of the ASGP2 and ErbB2/Neu proteins is necessary for their interaction. When the conditioned media from insect cells independently expressing ASGP2 ECD and Neu ECD were mixed, no co-immunoprecipitation of ASGP2 and ErbB2/Neu was observed. This is consistent with our observations that ASGP2 ECD expressed in either insect cells or COS monkey cells will neither bind to nor activate ErbB2/Neu when added exogenously to cultured mammalian cells that express this receptor (21). The reason for the requirement of co-expression for ASGP2-ErbB2/Neu complex formation is presently unclear. The simplest explanation is that high concentrations of ASGP2 and ErbB2 are necessary for complex formation, a condition which is met in membranes and in cellular compartments, but not by the addition of soluble ligand to cells. This condition may be satisfied in the DHFR/G8 receptor activation experiments by intercalation of ASGP2 into the membranes in the presence of low concentrations of detergent. Once formed, the complex is very stable and resistant to dissociation

during the immunoprecipitation procedures. Other phenomena, not necessarily mutually exclusive, may also contribute to the ineffectiveness of ASGP2 to act as a diffusible ligand. For example, the presence of an unknown cell surface factor or the post-translational modification of either ASGP2 or ErbB2/Neu may impose constraints on the association of the mature ligand with the cell surface receptor. It should be noted that our observations do not rule out the possibility that soluble ASGP2 may act as a diffusible ligand under conditions not yet examined. In that regard it is relevant to note that ASGP2 is secreted in a soluble form from a number of tissues, including lactating mammary gland and intestine (13). In rat milk ASGP2 is present at a 10-fold higher concentration than EGF, normally considered the most abundant milk growth factor (22), and is present as a complex with the mucin ASGP1, a form we have not yet tested as a modulator of cellular ErbB2/Neu.

We conclude that in the ascites 13762 cells, ASGP2 interacts directly with ErbB2/Neu through its EGF1 domain, and contributes to the constitutive activation state of this receptor. The augmented ErbB2/Neu activity may in turn contribute to the autonomous growth properties or the malignancy of the ascites cells. A question that naturally arises is whether the sialomucin complex contributes to human breast cancer. In a preliminary study, sialomucin complex was found in approximately 70% of highly aggressive breast tumors from patient effusions, but in only 15% of ErbB2-positive solid tumors, perhaps suggesting a role in tumor progression (23). Further studies are in progress to substantiate these findings and to determine whether sialomucin complex may have significance in prognosis or as a target for therapy.

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11. Ascites 13762 adenocarcinoma cells (MAT-C1 subline) were grown intraperitoneally in Fischer 344 rats, and microvillar membranes were prepared under microfilament-depolymerizing conditions as described previously [K. L. Carraway, J. W. Huggins, R. F. Cerra, D. R. Yeltman, and C. A. C. Carraway, *Nature* **285**, 508 (1980); K. L. Carraway, R. F. Cerra, G. Jung, and C. A. C. Carraway, *J. Cell Biol.* **94**, 624 (1982)]. Microvilli (5.0 ml, ~15 mg/ml protein) were incubated for 30 min at 4°C in 5 mM glycine, 2 mM EDTA, 2 mM dithiothreitol, pH 9.5, and then homogenized in a Dounce B homogenizer. After centrifugation at 10,000xg the membranes were harvested and washed using

centrifugation at 150,000xg for 1.5 hr. Microvillar membranes were solubilized in S buffer (0.2% Triton X-100, 150 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM Tris, pH 7.6) and centrifuged on 7-25% sucrose gradients in S buffer for 15 hr at 80,000xg and 4°C. Gradients were fractionated and prepared for SDS-PAGE and immunoblotting. Selected fractions were combined and analyzed by immunoprecipitation (12).

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13. Immunoprecipitations of rat ErbB2/Neu or its deleted variants were carried out with 1 μg of either Ab-3 or Ab-4 anti-ErbB2/Neu monoclonal antibodies (Oncogene Science) using 2 μg rabbit anti-mouse IgG secondary antibody (Zymed) to bind to Protein A-Sepharose. Immunoprecipitations of ASGP2 were carried out with 3 μl polyclonal anti-ASGP2 (7). Immunoblotting using anti-ErbB2/Neu Ab-3 (10), polyclonal anti-ASGP2 (7) or monoclonal anti-ASGP2 (13) was performed using either alkaline phosphatase or enhanced chemiluminescence for detection.

14. E. A. Rossi *et al.*, manuscript submitted for publication.

15. Sf9 insect cell growth and infections were carried out as previously described [P. M. Guy, K. L. Carraway III and R. A. Cerione, *J. Biol. Chem.* **267**, 13851 (1992); K. L. Carraway III *et al.*, *J. Biol. Chem.* **269**, 14303 (1994)]. Construction of recombinant baculoviruses will be described elsewhere [E.A. Rossi, *et al.*, manuscript in preparation]. In co-expression experiments where a single protein was expressed as a control, wild type baculovirus was used as the co-infecting virus. Sf9 cell lysis was performed using an NP-40 lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1 mM orthovanadate, 100 μM leupeptin, 20 KIU/ml aprotinin, 1 mM PMSF, 1 mM benzamidine). Lysates were cleared by centrifugation at 12,000xg for 15 minutes prior to immunoprecipitation.

16. K. L. Carraway III, unpublished observations.

17. Infections of High Five cells were performed with $2x10^6$ cells/well of 12-well tissue culture dishes and incubation at 27°C. Cells were incubated for 24 hours with baculovirus containing DNA encoding Neu ECD and the ASGP2 deletion mutants at a multiplicity of infection between 5 and 10 for each virus. The media were replaced with Excell 405 serum free media (JRH Biological). The conditioned media were collected after an additional 32 hours and clarified by centrifugation at 12,000xg. An equal volume of 2X RIPA buffer was added prior to immunoprecipitation.

18. E. A. Rossi and K. L. Carraway, unpublished observations.

19. ASGP2 was purified to homogeneity by CsCl density gradient centrifugation [S. R. Hull, Z. Sheng, O. A. Vanderpuye, C. David, and K. L. Carraway, *Biochem. J.* **265**, 121 (1990)] followed by non-reducing SDS-PAGE (8). Microvilli were extracted with Triton X-100 under microfilament stabilizing conditions and centrifuged to remove microfilaments. The Triton supernatants were fractionated by CsCl density gradient centrifugation to yield purified heterodimeric sialomucin complex containing ASGP1 and ASGP2. The complex was subjected to SDS-PAGE under non-reducing conditions, and the ASGP2 band was excised and electroeluted.

20. DHFR/G8 cells were suspended in 20 mM HEPES/Na, pH 7.4, 1 mM PMSF, 0.1 mM pepstatin, 1 mM benzamidine, 20 KIU/ml aprotinin, incubated on ice for 15 minutes, and homogenized (15 strokes) in a Dounce B homogenizer. The homogenate was centrifuged at 1500xg for 10 min at 4°C and the supernate was transferred to Eppendorf tubes. Membranes were pelleted at 15,000xg for 15 min at 4°C, washed once in disruption buffer, and stored at -20°C in disruption buffer with 50% glycerol. For phosphorylation assays, membranes were washed once with and resuspended in buffer A (10 mM HEPES/Na, pH 7.4, 20 mM $MgCl_2$, 100 μM leupeptin, 20 KIU/ml aprotinin, 1 mM PMSF, 1 mM benzamidine), and ASGP2 or EGF in buffer A containing 0.02% Triton X-100 was added and the mixture incubated for 2 hours at room temperature. The membranes were phosphorylated for 10 minutes at room temperature in the absence or presence of 0.2 mg/ml

poly(glu$_4$tyr) with the addition of 5 μCi/reaction of [γ-^{32}P]ATP. Samples were solubilized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) for immunoprecipitation or prepared for SDS-PAGE. Label incorporated into the poly(glu$_4$tyr) substrate was analyzed by electrophoresis, autoradiography and densitometry.

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24. These studies were supported in part by American Cancer Society fellowship PF-3964 and a grant from Massachusetts Department of Public Health Breast Cancer Program (KLCIII), NIH grant CA52498 and a grant from the American Cancer Society BE-71064 (KLC), and by the Sylvester Cancer Center of the University of Miami (CA14395).

Figure Legends

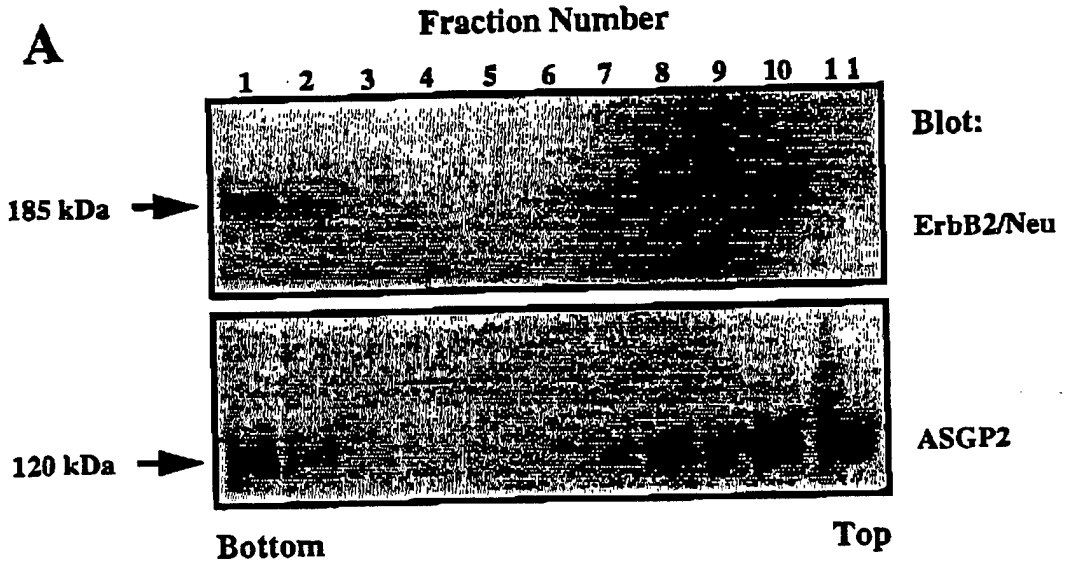
Fig. 1. Association of ASGP2 and ErbB2/Neu in 13762 cells. **(A)** Sedimentation of ASGP2-ErbB2/Neu complexes from 13762 cell microvillar membranes. Triton solubilized microfilament-depleted microvilli were subjected to velocity sedimentation (9), and fractions were analyzed by immunoblotting with anti-ErbB2/Neu (upper panel) or anti-ASGP2 (lower panel). Fractions 1 and 2 at the bottom of the tube represent the actin-associated signal transduction particle described previously (12), while fractions 8-11 at the top represent smaller membrane protein complexes not associated with the particle. **(B)** Co-immunoprecipitation of ASGP2 and ErbB2/Neu. Fractions 8-10 from (A) were pooled and immunoprecipitated with either polyclonal anti-ASGP2, monoclonal anti-ErbB2/Neu or control antibodies (13), as indicated. Precipitates were immunoblotted with anti-ErbB2/Neu (left three lanes) or anti-ASGP2 (right three lanes) after SDS-PAGE. Microvillar membranes were included as positive controls for blotting (lanes marked None).

Fig. 2. Association of ASGP2 with ErbB2/Neu when co-expressed in the same cell. **(A)** Specific association of ASGP2 with ErbB2/Neu. Sf9 insect cells were infected with baculovirus encoding EGF receptor, ErbB2/Neu or transmembrane $ASGP2_{tm}$ alone, or $ASGP2_{tm}$ together with each of the receptors, as indicated. Lysates from cells were immunoprecipitated with the indicated anti-receptor monoclonal antibodies and precipitates were blotted with monoclonal anti-ASGP2. Ponceau Red staining of the filter indicated that there were identical levels of ErbB2/Neu and EGF receptor in immunoprecipitates (16). **(B)** The extracellular domain of ErbB2/Neu is necessary for its interaction with ASGP2. Sf9 cells were co-infected with baculovirus encoding $ASGP2_{tm}$ and the illustrated ErbB2/Neu constructs (upper panel), and the co-immunoprecipitation assay was used to assess association (lower panel). Full-length rat Neu, NTK and MS-N were immunoprecipitated with Ab-3 which recognizes the intracellular domain, and Neu ECD was

immunoprecipitated with Ab-4 which recognizes the extracellular domain. The two lanes at the far right represent the immunopreciptation from lysates of cells expressing $ASGP2_{tm}$ alone using Ab-3 and Ab-4, respectively. Ponceau Red staining of the entire filter indicated that there were similar levels of the different ErbB2/Neu constructs in immunoprecipitates (16).

Fig. 3. Secretion of a complex of ASGP2 and ErbB2/Neu extracellular domains from co-expressing cells. **(A)** High Five insect cells were infected with virus encoding the extracellular domain of ErbB2/Neu alone (Neu ECD, lanes A), virus encoding the extracellular domain of ASGP2 alone (ASGP2 ECD, lanes B) or co-infected with the two viruses together (lanes C). Equal volumes of the conditioned media for the Neu ECD and ASGP2 ECD individual infections were also mixed (lanes D). Conditioned media and immunoprecipitates using anti-ErbB2/Neu monoclonal Ab-4 or polyclonal anti-ASGP2 were blotted with anti-ASGP2 after SDS-PAGE. **(B)** The EGF1 domain of ASGP2 is necessary for its interaction with ErbB2/Neu. High Five cells were co-infected with baculovirus encoding Neu ECD and the illustrated ASGP2 constructs (upper panel), and association was measured by co-immunoprecipitation (lower panel, left) from the conditioned media (lower panel, right) by immunoblotting with polyclonal anti-ASGP2.

Fig. 4. Stimulation of ErbB2/Neu tyrosine kinase activity in DHFR/G8 membranes by purified ASGP2. **(A)** Stimulation of ErbB2/Neu phosphorylation. Partially solubilized membranes were treated with 100 nM EGF or 200 nM purified ASGP2, incubated with [γ-^{32}P]ATP, and then cleared TX-100 lysates were immunoprecipitated with anti-ErbB2/Neu. An autoradiogram of the dried gel is depicted. **(B)** Untreated and ASGP2-treated membranes were used to phosphorylate the synthetic substrate poly(Glu_4Tyr) in the presence of [γ-^{32}P]ATP.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ASGP2
Bottom
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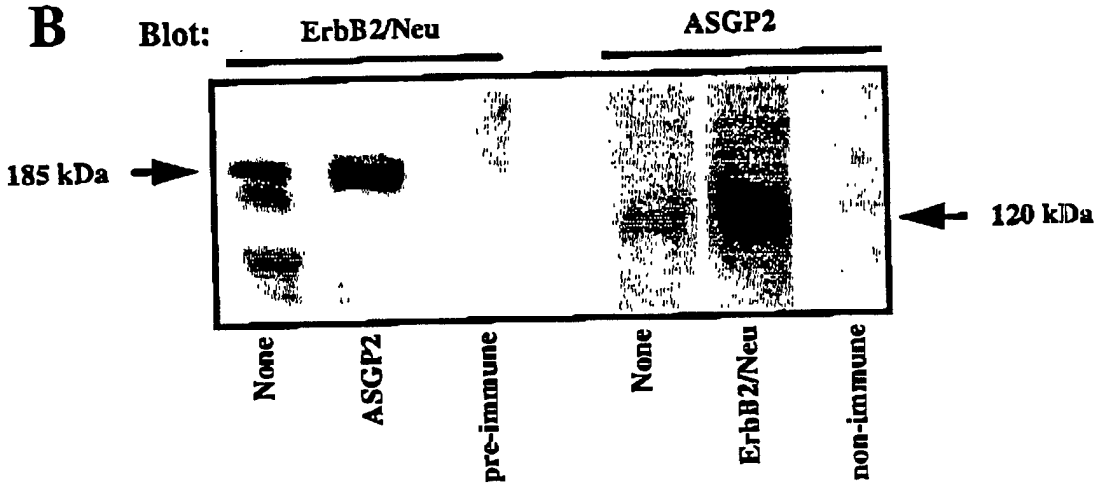
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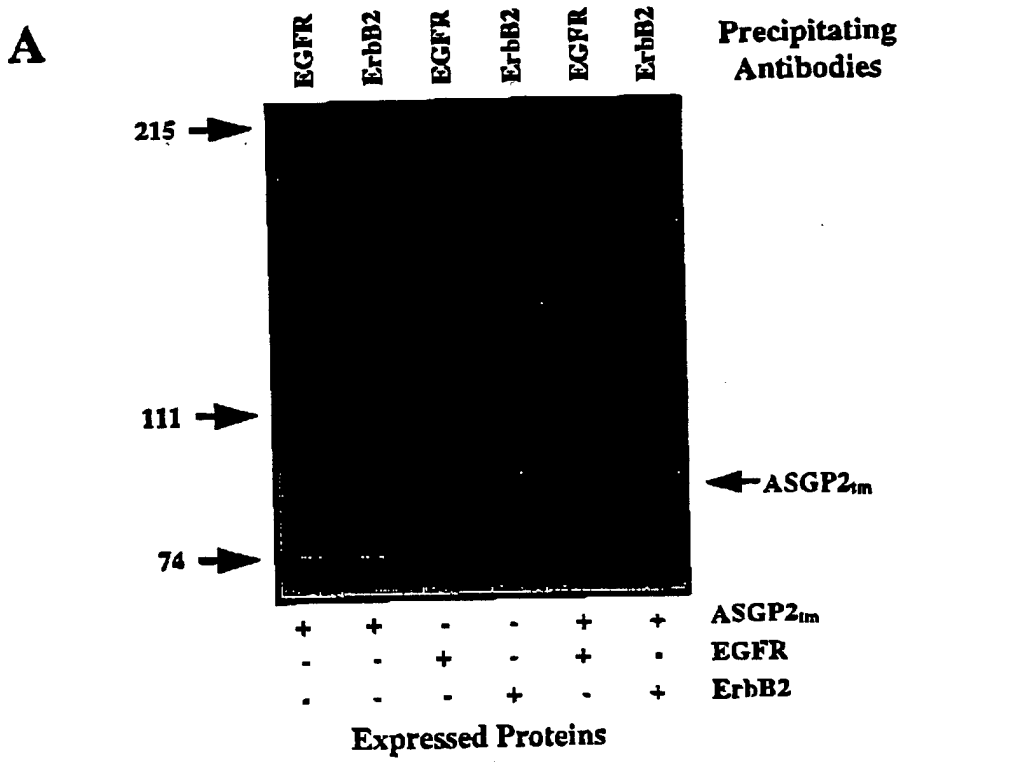
Figure 1

Figure 2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Construct
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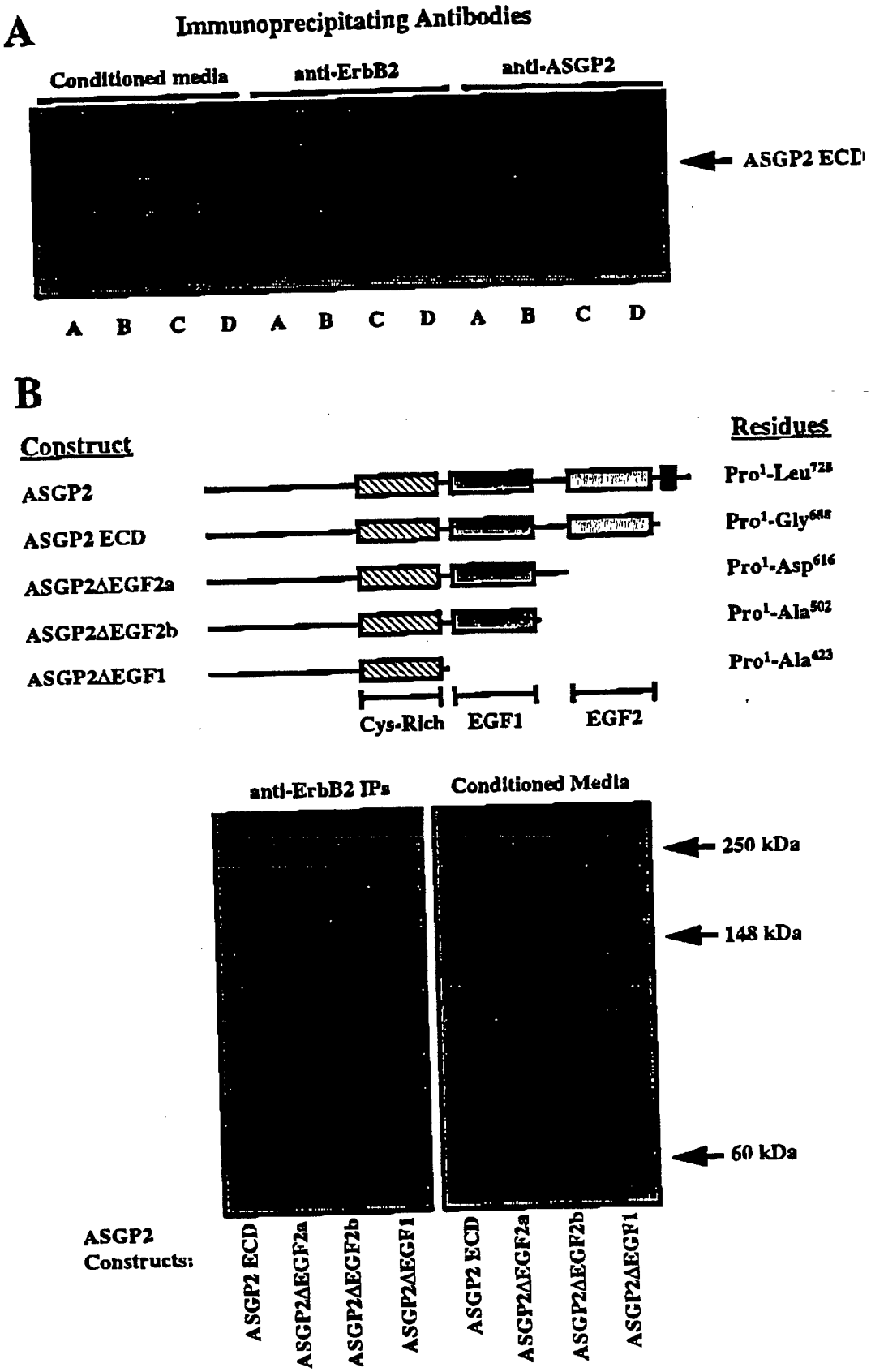
Figure 3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anti-ASGP2
ASGP2 ECD
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ASGP2 ECD
ASGP2ΔEGF2a
ASGP2ΔEGF2b
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Pro1-Ala423
Cys-Rich
EGF1
EGF2
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Conditioned Media
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148 kDa
60 kDa
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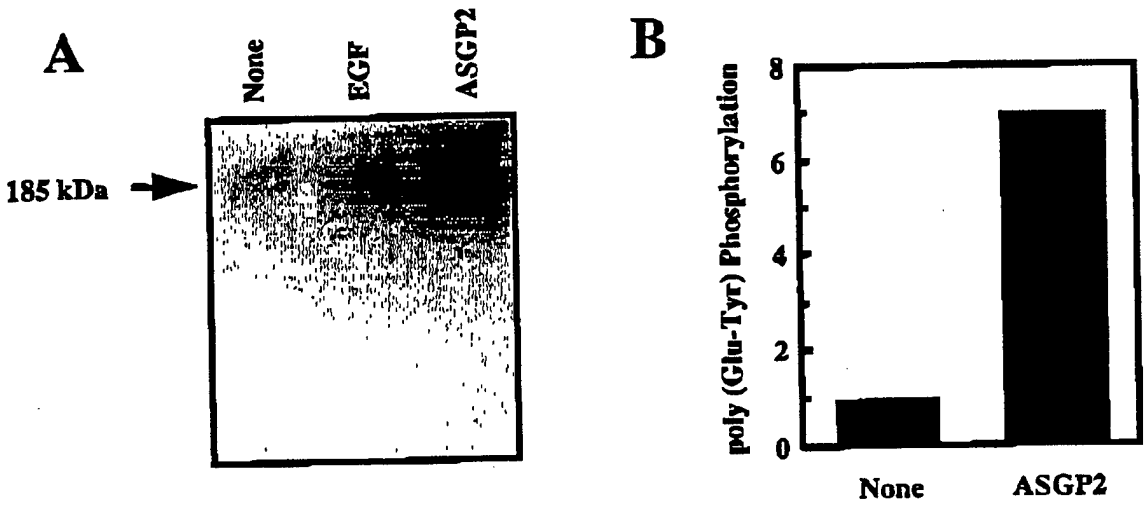
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None
EGF
ASGP2
185 kDa
B
poly (Glu-Tyr) Phosphorylation
8
6
4
2
0
None
ASGP2

Figure 4